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Immunological Determination of Human Fetal Hemoglobin

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A new method is described for the immunological determination of human fetal hemoglobin (HbF) using specific antiserum. Whole blood is pretreated with an alkaline reagent to denature and remove adult hemoglobin (HbA) and other non hemoglobin proteins, leaving HbF in solution. HbF is then determined by single radial immunodiffusion. The specificity, accuracy and high sensitivity of the method were found to be satisfactory. The HbF concentration in normal individuals and patients was determined by this method.

Es wird ein neues immunochemisches Verfahren für die Bestimmung von menschlichem fötalen Hämoglobin mittels spezifischen Antiserums beschrieben. Das Probenblut wurde mit einem alkalischen Reagenz behandelt, mit dem Erwachsenen-Hämoglobin und andere Eiweiße außer dem fötalen Hämoglobin denaturiert und von der Lösung ausgeschlossen wurden. Dann wurde der Gehalt des fötalen Hämoglobins in der Lösung durch die einfache radiale Immunodiffusion bestimmt. Die Spezifität, Genauigkeit und Empfindlichkeit der Methode war genügend. Fötales Hämoglobin von Gesunden und Kranken wurde mit der vorliegenden Methode bestimmt.

It is well known that HbF increases in some hematologic diseases, including leukemia (1, 2, 3), hypoplastic anemia (4, 5), and thalassemia (6, 7). The clinical significance of this observation and the mechanism of the change from fetal to adult hemoglobin synthesis is of interest.

The one minute alkali-denaturation method of Singer et al. (8) has been widely used for the determination of HbF, but there are some problems concerning the specificity of this determination. Many other methods for the determination of HbF, such as chromatographic (9, 10), spectrographic (11) and amino acid analytical methods (12) have been reported, but these procedures are rather complex and their accuracy is not satisfactory, especially at low concentrations of HbF.

For these reasons, we have attempted to develop a new method for the determination of HbF based on an antigen-antibody reaction which is highly specific and sensitive compared with Singer's method. The level of HbF in some blood specimens from normal individuals and some patients was determined by this new method.

Experiments and Results

Preparation of Antigen

HbF solution was prepared from cord blood by Chernoff's method (13), and further purified by Amberlite CG-50 column chromatography according to the method of Allen et al. (14) to remove non-hemoglobin proteins (15), which would interfere with the production of specific antiserum against HbF. A partially purified solution of HbF (5 ml, 40 mg/ml) was applied to a column (3 × 30 cm) of Amberlite CG-50 equilibrated with 0.04 mol/l sodium phosphate buffer, pH 6.9 containing 0.01 mol/l KCN, and eluted with the same buffer at a flow rate of 40 ml per hour.

Optical density of fractions was measured at 415 nm as shown in Figure 1. Under these conditions, fraction I, II and III were obtained. Non-hemoglobin protein in these fraction was identified electrophoretically (Fig. 2) and verified using a precipitin reaction with antisera against non-hemoglobin proteins. Electrophoresis was carried out according to the method of Rosenbaum (16). The non-hemoglobin protein was prepared by the method

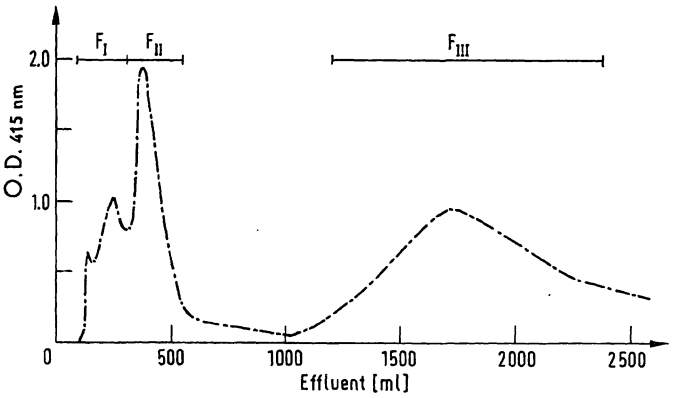

Fig. 1
Chromatography of HbF

Column:	3 × 30 cm, Amberlite CG-50
Buffer:	0.04 mol/l sodium phosphate buffer, pH 6.9 Na+ 0.05 mol/l
Sample:	200 mg of HbF (about 5 ml)
Flow rate:	40 ml/h

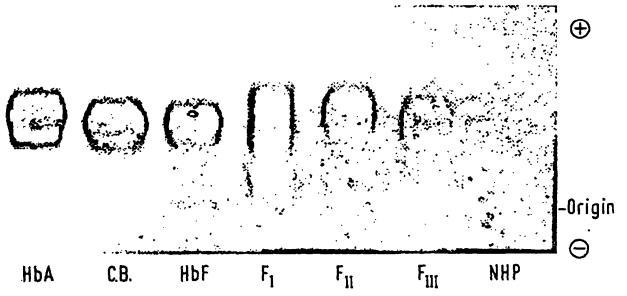

Fig. 2
Electrophoretic pattern of various hemoglobin solutions

Supporting media:	Cellulose acetate membrane
Buffer:	Tris-EDTA-borate buffer pH 9.0
Current time:	0.6 mA/cm 40 min
Staining:	Ponceau 3R
Sample HbA:	Hemolysate of normal adult blood
C.B:	Hemolysate of cord blood
HbF:	HbF purified by Chernoff's method
F_I—F_{III}:	Fractions of HbF on Amberlite CG-50
NHP:	Non hemoglobin protein

of WINSLOW et al. (17). All these fractions contained HbF but the non-hemoglobin protein was found only in fraction I. Fraction II and III were collected as purified HbF and used as the antigen.

Production of Antisera

The Antigen solution (HbF: about 20 mg/ml) was mixed with an equal volume of FREUND's complete adjuvant and 2 to 2.5 ml of the mixture containing 20 to 25 mg of HbF was injected subcutaneously into a rabbit's back once a week for 4 to 6 weeks. The rabbits were periodically bled from marginal ear veins and the sera were collected for precipitin tests. At 10 to 14 days after the last injection, the blood was obtained from the Arteria carotis. The antigen-antibody reaction was studied with the double diffusion technique in agar according to the method of OUCHTERLONY (18). The antigens tested were normal human serum, non-hemoglobin proteins, HbA_0, HbA_2, hemolysate of cord blood, HbF prepared according to CHERNOFF's method, and the chromatographed fractions mentioned previously. HbA_0 and HbA_2 were prepared according to the method of WINSLOW et al. (17).

The result is shown in Figure 3. The precipitin lines showed that there were no antigenic differences with respect to the antiserum used between HbA_0 and HbA_2, and also between HbF, its fractions and hemolysate of cord blood. The precipitin line due to HbA_0 (or HbA_2) and that due to HbF, however formed a "spur", indicating partial antigenic identity of HbA_0 and HbF. This appears to be due to the α-chain which is common to both HbA_0 and HbF. Therefore, the antibody against α-chain in the antiserum was absorbed by purified HbA_2 solution since it was easily obtained free of HbF. After absorption of antiserum with HbA_2, the antiserum reacted only with HbF and its fractions, demonstrating that it was specific for HbF as indicated in Figure 4.

Immunological determination HbF

An HbF solution prepared by means of CHERNOFF's method as mentioned previously was serially diluted with saline and used for standardization. The gels for immunodiffusion were prepared in Petri dishes with 0.4 ml of antiserum mixed with 9.6 ml of 1.2% agarose-saline solution. Single radial immunodiffusion was carried out according to the method of MANCINI et al. (19), and the squared diameters of precipitin rings were plotted as a function of antigen concentration.

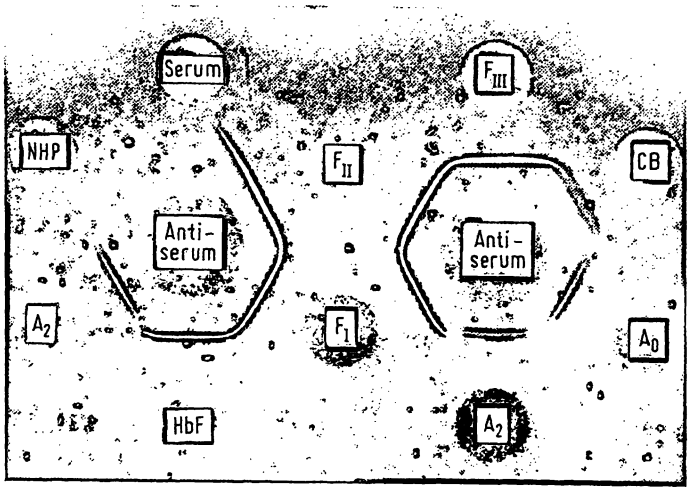

Fig. 3
Precipitin reaction of anti-HbF serum in agar gel

Antiserum:	Rabbit antiserum against HbF
NHP:	Non hemoglobin protein
C. B.	Cord blood hemolysate
FI-III:	Fraction of HbF from Amberlite CG-50
A_0:	HbA_0
A_2:	HbA_2
HbF:	HbF purified by CHERNOFF's method
Concentration of proteins:	0.5 mg/ml

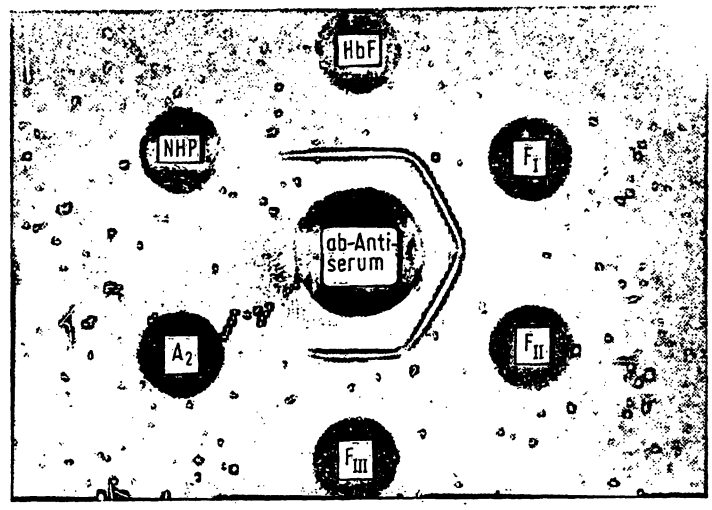

Fig. 4
Precipitin reaction of absorbed anti-HbF serum in agar gel
Nomenclature is the same as Fig. 3
ab-Antiserum: Anti-HbF serum absorbed with HbA_2

A linear relation between antigen concentration and the squares of the diameters of precipitin rings was observed as shown in Figure 5. The sensitivity of this method was 20 mg HbF/ml and the calibration range was 20 to 600 mg/ml.

Interference with the HbF-anti HbF antibody reaction by HbA

Though the specific antiserum reacted only with HbF, it was found that the precipitin rings yielded by hemolysates from normal individuals or patients were so obscure that the measurement of the diameter of rings was difficult. In order to understand the reason for this obscurity of the precipitin ring, various amount of purified HbA, non-hemoglobin proteins or serum were

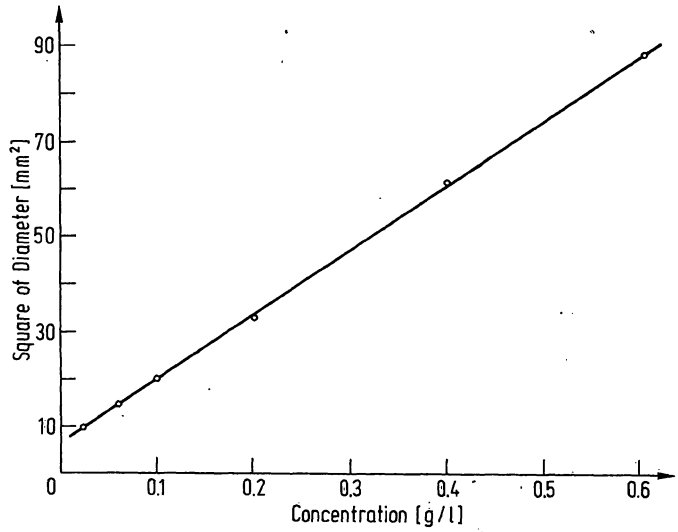

Fig. 5
Calibration curve of HbF by precipitin reaction
Agar gel in the Petri dish contained of 0.4 ml antiserum and 9.6 ml 1.2% Agarose-saline. Each standard solution of HbF is subjected to immunodiffusion for 24 to 36 hours

added to the solution of definite amounts of purified HbF, and these mixtures were subjected to the single radial immunodiffusion procedure. It was evident that HbA interfered with the precipitin reaction and the obscurity of the precipitin rings was observed when the content of HbA in the mixtures was more than twice that of HbF. The non-hemoglobin proteins and serum did not affect the precipitin ring. Why HbA interferes with the precipitin reaction of HbF is not clear. Thus it is necessary to remove HbA from the sample hemolysates prior to immunodiffusion.

Determination of HbF

Pretreatment of the blood specimen

Blood was obtained from normal individuals and patients using either 3.8% sodium citrate or heparin as anticoagulant. Prior to immunodiffusion, the blood specimens were treated directly with alkaline reagent according to CHERNOFF's method (13) to remove HbA and proteins other than HbF; 2 volumes of whole blood were mixed with 16 volumes of $^1/_{12}$ mol/l NaOH solution for various times at 20°C and then the reaction was stopped with 9 volumes of saturated $(NH_4)_2SO_4$ solution containing 9.5 ml of conc. HCl per l.

After a few minutes, the reaction mixtures were centrifuged at 3000 r. p. m. for 10 min to obtain the supernatant as shown in Figure 6. 4 μl of the supernatant was subjected to single radial immunodiffusion for 24 to 36 h and the amount of HbF (g/l) was calculated from the calibration curve.

When the specimen was reacted with alkaline reagent for more than one minute, the precipitin rings were clear, showing that most of HbA in blood was removed and its inhibitory effect of formation of precipitin ring was eliminated. Moreover the longer the reaction time with alkaline reagent, the smaller the precipitin ring. This indicated that HbF was also denatured by the alkaline reagent though its denaturation rate was very low. When the logarithms of the amounts of HbF estimated at various intervals were plotted against the reaction time, straight lines were obtained in all specimens containing various amount of HbF as shown in Figure 7. This observation indicated that the alkali-denaturation of HbF was "pseudo" first order reaction. By extrapolating the straight line to zero time, the quantity of HbF in the specimens can be estimated. The slope of the lines showed practically the same value

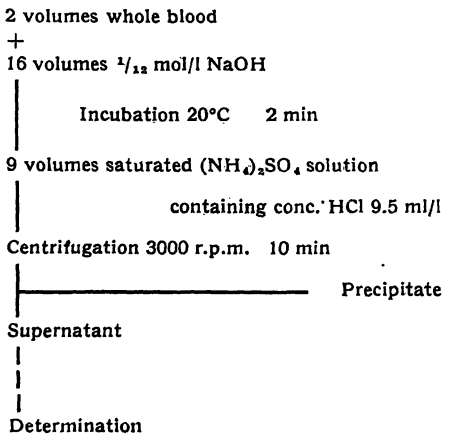

Fig. 6
Alkali-treatment of blood specimen for determination of HbF

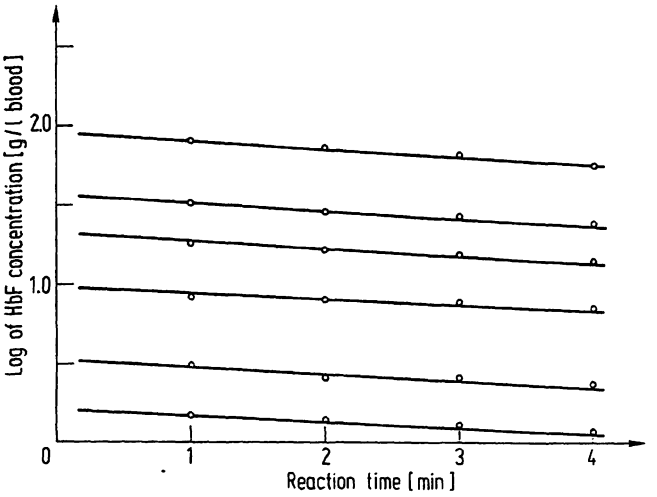

Fig. 7
Rate of alkali denaturation of HbF in blood
The bloods which contained various amounts of HbF were prepared by mixing normal adult blood and cord blood. The procedure for the alkali denaturation is the same as in Fig. 6 except for the reaction time
$k = 3.50\,(\pm\,0.14)\,.\,10^{-2}\ [min^{-1}]$ ($\bar{x} \pm s$)

regardless of the concentration of HbF in the range from 0.03 to 13.5%. The mean value of the slope was

$$3.50\,(\pm\,0.14) \times 10^{-2}/\text{min} \qquad (\pm\ \text{S. E.})$$

From this value, the following equation was obtained.

$$\log x_0 = \log x_t + 3.5 \times 10^{-2} \cdot t \quad \text{or} \quad x_0 = 10^{3.5 \times 10^{-2} \times t} \cdot x_t$$

Where x_0 is the amount of HbF (g/l) at zero time and x_t is that at t min.

Practical procedure for determination of HbF

The amount of HbF was determined using the same procedure described above and calculated by the following equations.

$$a = 10^{3.5 \times 10^{-2} \times 2} \cdot x_2 = 1.175 \cdot x_2$$

$$\text{HbF (\%)} = \frac{a \times 13.5}{\text{total hemoglobin}} \times 100$$

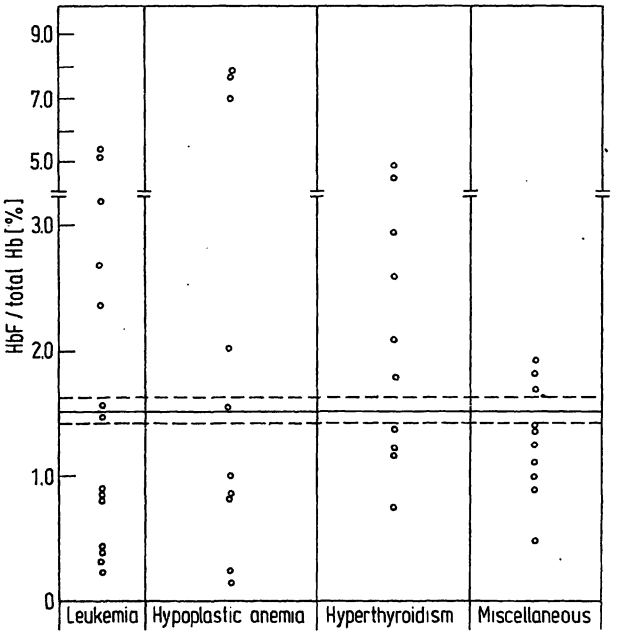

Fig. 8
HbF level in some diseases
The dotted lines define the values for normal adults:
$\bar{x} \pm s = 1.53 \pm 0.06\%$

Where, a is the concentration of HbF at zero time, x_2 is the concentration of HbF measured after 2 min of alkali treatment, HbF % is the percent total hemoglobin measured by the cyanmethemoglobin method (20), and 13.5 is the reagent dilution factor. The time of alkali treatment was chosen such that satisfactory amounts of HbA would be eliminated, that the loss of HbF should be avoided and also that the reaction time should be as short as possible: 2 min for the alkali treatment satisfied these conditions. The reproducibility of the determination was examined by repeated testing of the same sample, giving a test error of $\pm$ 3%.

HbF levels in some diseases

The percent concentration of HbF in total hemoglobin was determined using this method for blood samples from normal individuals and patients with leukemia, hypoplastic anemia, hyperthyrodism and other diseases. The results are shown in Figure 8. In some cases, high values of HbF were observed, but the clinical significance is not yet clear.

Discussion

For this method, the preparation of monospecific antiserum against HbF is essential. Hitherto, HbF as an antigen solution was purified by means of CHERNOFF's method, but this method fails to remove all non-hemoglobin proteins which are quite antigenic compared with HbF, so that the antisera obtained contained antibodies against non-hemoglobin proteins as well as HbF. For this reason, HbF was further purified on an Amberlite CG-50 column to eliminate non-hemoglobin proteins. The purified HbF preparation, which was practically free of non-hemoglobin proteins, was used as an antigen for immunization of rabbits. The antiserum thus obtained reacted with HbF and HbA only, but did not show any reaction with non-hemoglobin proteins. This indicated that this antiserum contained only antibodies against the α and γ-chains. The antibodies against the α-chain were then absorbed by HbA_2. This absorbed antiserum was found to be highly specific for HbF, and the antibody titer was satisfactorily high.

The blood specimens were treated directly by the same alkali-denaturation method as that used for the preparation of HbF, for the following reasons. First, HbA interfered with the precipitin reaction between HbF and its antibodies, so that HbA had to be removed from the specimens prior to immunodiffusion. Second, since the purified HbF used as the immunogen was prepared using the alkali-denaturation procedures, there may be some effect on the antigenicity of HbF.

Therefore, the blood specimens were also treated by the same alkali-denaturation procedure on the expectation that the difference between the antigenic structure of the immunogen and antigen would be minimized. Third, instead of hemolysate, whole blood was directly treated with the alkali reagent, eliminating the time required for preparation of hemolysate.

For the determination of HbF, SINGER's method has been most widely used. Compared with SINGER's method, the present method seems to be more specific for HbF because of the specificity of the antisera against HbF; moreover in SINGER's method, it is possible that some contaminants such as CO-HbA and other substances which are resistant to the alkali reagent might contribute to the absorption at 540 nm. Another advantage of this method is that the time necessary for preparation of hemolysate in SINGER's method is eliminated. The sensitivity of this method is about 20 times greater than that of SINGER's method, since it is possible to determine as little as 20 mg of HbF per l. The values of HbF determined by this method were compared with those by SINGER's method with the same samples. The present method gave results about 10% higher than SINGER's method. This difference is greater than expected even taking into account the fact that the amount of HbF degraded during 1 min of the alkali treatment in SINGER's method is about 4 percent (21).

A small amount of the alkali-degraded fragment of HbF may remain in the supernatant. If the absorption at 540 nm of the this fragment were lower than that of HbF, but its antigenicity were retained, the lower value by SINGER's method would be accounted for. More precise studies are, however, necessary to confirm this conjecture.

Both this new method and SINGER's method rely on the assumption that the denaturation reaction of HbF by the alkaline reagent is approximately a first order reaction and the rate of the reaction is constant regardless of the concentration of HbF. SINGER et al. (22) reported a different rate of denaturation in some diseases, although these seemed to be rather exceptional. In 1953, CHERNOFF developed the immunological determination method of HbF based on the relationship between the appearence time of the precipitin rings and the concentration of HbF in precipitin tubes. However, when the concentration of HbF in specimens was high, the appearence time of precipitin rings was so short that the error of determination became large, and when the concentration of HbF was low, the appearence time was over 100 min (7). For these reasons, the CHERNOFF's method might not be satisfactory for routine determination. The present method utilizing immunodiffusion is more convenient because the reaction time is short and the determination of 30 to 40 specimens is possible using only one gel, though 24 to 36 h are needed for the immunodiffusion. KOHN et al. (23) reported recently a modified single radial immunodiffusion method for the determination of HbF. In this method, hemolysates, the concentration of which was adjusted to about 100 g/l, were used, as in SINGER's method, and also Nigrosin staining of the precipitin rings was necessary for detection against the HbA background and to increase the sensitivity. In the present method, HbF can be determined with satisfactory sensitivity without resorting to staining or the preparation of an hemolysate.

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